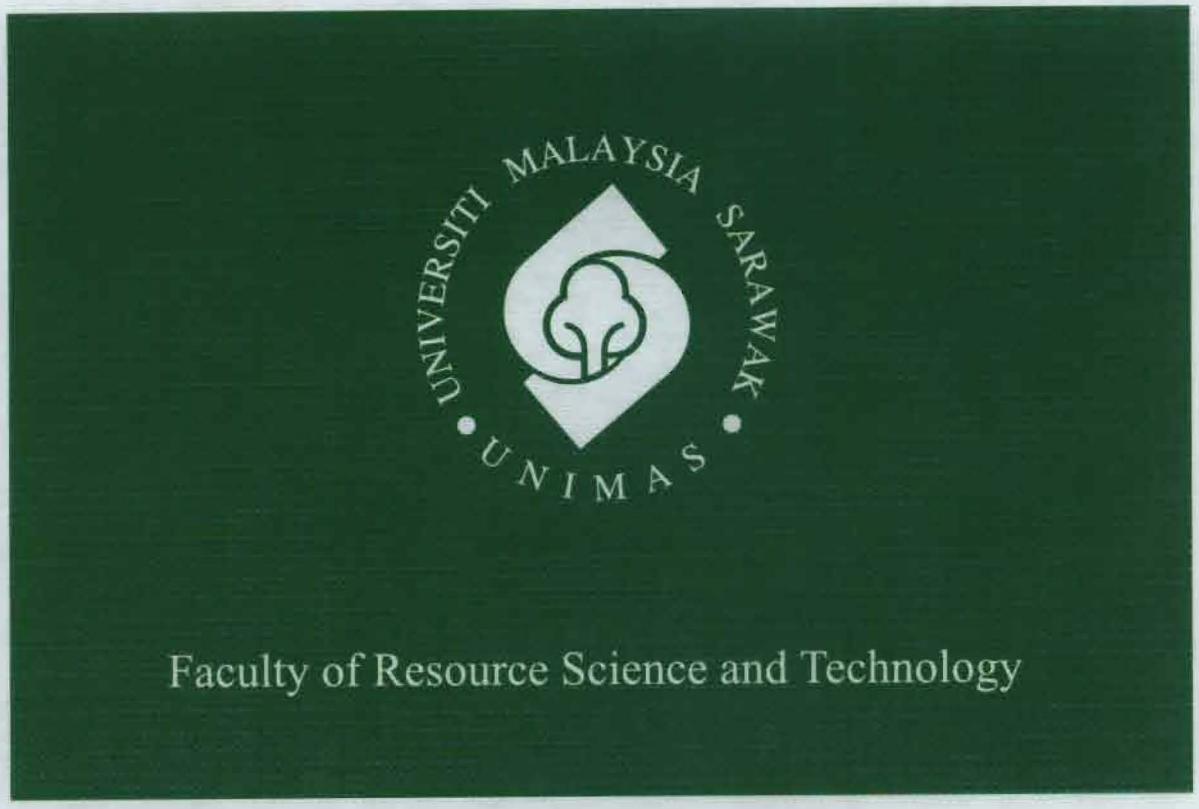

EFFECTS OF CASTOR OIL ON *Escherichia coli* K011 GROWTH AND ACTIVITIES DURING FED-BATCH FERMENTATION

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Bachelor of Science with Honours
(Biotechnology Resource)
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EFFECTS OF CASTOR OIL ON *Escherichia coli* K011 GROWTH AND ACTIVITIES DURING FED-BATCH FERMENTATION

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This project is submitted in partial fulfilment of requirement for the degree of Bachelor of Science with Honours

(Resources Biotechnology)

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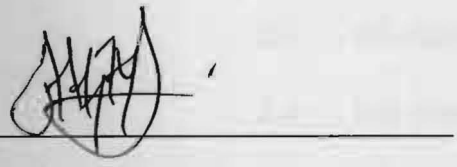
DECLARATION

I hereby declare that this Final Year Project report 2014 entitled "Effects Of Castor oil On *Escherichia coli* K011 Growth And Activities During Fed-Batch Fermentation" is based on my original work except for quotations and citations which have been dully acknowledged also, declare that the work referred in this project has been submitted in support of an application for another degree qualification of this or any other university or institution of higher learning.



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LIST OF ABBREVIATIONS

E. coli K011	*Escherichia coli* K011
HPLC	High Performance liquid Chromatography
PBS Buffer	Phosphate Buffered Saline
LB broth	Luria Bertani Broth
CFU	Colony Forming Units
kb	kilobase
°C	Celsius
rpm	Revolution per minute
%	Percentage
g	Gram
µl	Microliter
ml	Milliliter
spp.	Species
wt	Weight
g/l	Gram per liter
hr	Hours

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Effect of Castor oil on *Escherichia coli* K011 Growth and Activities during Fed-Batch Fermentation

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ABSTRACT

The current depletion of fossil fuels, is causing excessive burdens in fuels supplied. This phenomenon has resulted in the rising price of fossil fuels globally. To resolve this issue, non-petroleum based fuels such as bioethanol is chosen as a primary alternative solution for fossil fuels energy. Traditionally, bioethanol is obtained from a process called fermentation. However during fermentation, ethanol is accumulated in the fermentation that effects growth and activity of the fermented bacteria. In this study, the production of ethanol was performed using *Escherichia coli* K011, and castor oil will be introduced into the fermentation vessel as a selective separator to absorb and separate the ethanol from the broth. The variable sugar added in this experiment are glucose and xylose. During the study, the detection of bioethanol are analyzed using High-Performance Liquid Chromatography (HPLC). The presence of castor oil sample, was determined by comparing with control sample. The experiment was conducted in glucose and xylose concentration level (5%, 10%, 20% and 30%) and the growth *E. coli* K011 will be monitor and calculate from 0 to 84 hr fermentation (4 days). According to the results obtained, the best concentration of glucose/xylose and effective for production of ethanol during fed-batch fermentation was 10% sugar concentration with highest ethanol production at 45.80 g/l for castor oil and 46.53 g/l for control broth respectively with the optimum glucose and xylose utilization. The result gained from experiment are expected to give a positive impact in the production of ethanol through fed-batch fermentation.

Key word: Bioethanol, Fermentation Activities, Castor oil, *Escherichia coli* K011, High- Performance Liquid Chromatography (HPLC), Fed-Batch Fermentation.

Pengurangan bahan api fosil, disebabkan permintaan berlebihan dalam bahan api yang dibekalkan. Fenomena ini telah menyebabkan kenaikan harga bahan api fosil di peringkat global. Untuk menyelesaikan isu ini, bahan api bukan petroleum seperti bioetanol dipilih sebagai penyelesaian alternatif untuk penghasilan tenaga. Secara tradisinya, bioetanol diperolehi dari proses yang dipanggil penapaian. Walau bagaimanapun semasa penapaian, etanol terkumpul dan memberi kesan kepada pertumbuhan dan aktiviti bakteria. Dalam kajian ini, pengeluaran etanol dilakukan dengan menggunakan *Escherichia coli* K011, dan minyak kastor akan diperkenalkan ke dalam sistem penapaian sebagai pemisah terpilih untuk menyerap dan mengasingkan etanol. Bahan pembolehubah yang ditambahan dalam eksperimen ini adalah glukosa dan xylose. Dalam kajian itu, bioethanol akan dikesan dan diproses menggunakan Berprestasi Tinggi Cecair Kromatografi (HPLC). Kehadiran sampel minyak kastor, akan ditentukan dengan membandingkan dengan sampel kawalan . Eksperimen ini dijalankan dalam tahap kepekatan yang berbeza (5 %, 10 %, 20 % dan 30 %) dan pertumbuhan *E. coli* K011 dipantau dari 0 - 84 jam penapaian (4 hari). Hasil keputusan yang diperolehi , kepekatan yang terbaik glukosa / xylose untuk pengeluaran etanol penapaian pemakanan berkumpulan adalah pada kadar 10% kepekatan gula dengan pengeluaran etanol tertinggi (45.80 g/l untuk minyak kastor ; 46.53 g/l untuk kawalan) dengan penggunaan optimum glukosa dan xylose . Hasil yang diperolehi daripada eksperimen ini memberi kesan positif kepada penghasilkan etanol melalui penapaian pemakanan berkumpulan.

Kata Kunci : Bioetanol, Aktiviti Penapaian, Minyak Castor, *Escherichia coli* K011, Cecair Kromatografi Berprestasi Tinggi (HPLC), Penapian Pemakanan Berkumpulan.

CHAPTER 1
INTRODUCTION

1.1 Introduction

The depletion of fossil fuels and the rising number of vehicles on the roads are among many factors why non-petroleum based fuels productions are being studied. One of the examples of non-petroleum based fuels is bioethanol. Currently, bioethanol are studied to supplement the inevitable depletion of nature fuels and reduce emission of greenhouse gas effect on earth (Ogunniyi, 2005). Besides that, the production of bioethanol is known to be inexpensive and environmental friendly.

Fermentation is still preferred method in ethanol production (Vincent *et al.,* 2011). The major problem in the production of ethanol is the accumulation of ethanol in fermentation broth. The accumulation of ethanol in fermentation will produce toxic effects that may affect *Escherichia coli* K011 performance. One possible method to solve the problem is by introducing castor oil into the fermentation broth.

Basically, castor oil contains almost 87.1% of ricinoleic acid in its composition (Offemen *et al.,* 2006). This fatty acid content has the capability to absorb ethanol, reduce the separation factor, and improve the process of fermentation (McKeon *et al.*, 2007; Offemen *et al.,* 2006). Ultimately this will positively effect ethanol productivity. This study was carried out to determine the effect of castor oil on *Escherichia coli* K011 growth and activities in fed-batch fermentation. The result for this experiment will improve the fed-batch fermentation production of ethanol.

1.2 Objectives

The objectives of this study are:

1. To determine the effects of castor oil on the fermenting activities of *Escherichia coli* K011 during anaerobic fermentation.
2. To study the effects of castor oil on *Escherichia coli* K011 growth during fed-batch fermentation.
3. To determine the best glucose concentration for effective ethanol production during the fed-batch fermentation.

CHAPTER 2

LITERATURE REVIEW

2.1 Castor oil

Castor oil is extracted from the seed of castor plants (Ogunniyi, 2005). Although identified as a type of vegetable oil, castor oil is not edible due to its toxicity. Castor plants are known as *Ricinus communis* (Ombrello, 1983). The name was taken from the Latin word due to the plant body resembles the ticks (Ombrello, 1983). Castor oil were identify as pale yellow in color. Upon extraction, it will turn clear with slightly yellowish although the skin is dark in color (Weise, 1983). As for composition, castor oil are more polar than conventional fats due to the present of ricinoleic acid. It contains triglycerides of 90% unsaturated fatty acid that comprises of ricinoleic acid, oleic acid, stearic acid, linoleic acid, and palmitic acid (Ombrello, 1983).

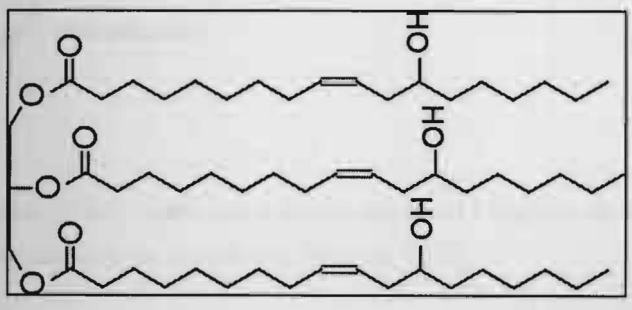

Figure 1: Structure of major component of castor oil (Cangemi *et al.*, 2008)

The presence of ricinoleic acid will produce unique chemical and physical properties in hydroxyl group at C-12 (Figure 1) (de lima da silva *et al.*, 2009). This hydroxyl group can create affinity between castor oil and ethanol at room temperature around 27- 33 °C. Other than ethanol, castor oil also dissolves easily in glacial acetic acid, chloroform, carbon sulfide and benzene (de lima da silva *et al.*, 2009).

Table 1 : Physicochemical parameter for the evaluation of castor oil (Ojediran *et al.*, 2011).

Parameters	Value
Refractive index	1.473-1.477
Acidity index	1.48
Saponification index	176-187
Iodine value	81-91
Specific gravity	0.9587

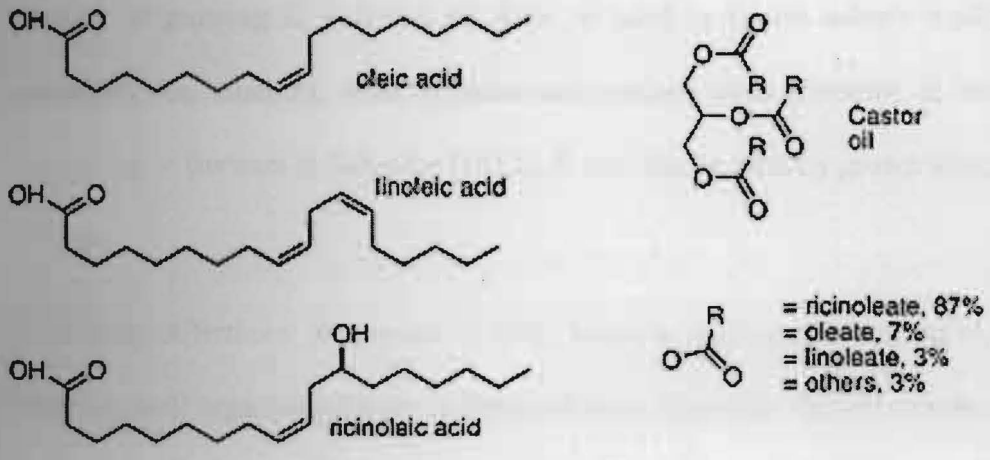

Figure 2: General structure of oleic, lauric and ricinoleic acids and a triglyceride showing the most important compound present in castor oil (Wender, 2010).

2.2 *Escherichia coli* K011

Escherichia coli is a gram-negative, rod-shaped, facultative anaerobe and can be found in human large intestinal and warm blooded animals (Singleton, 1999). This bacterium belongs to the family of *Enterobacteriaceae* (Keating, 2009). The genome of *E. coli* is circular with the size of about 4500- 4900 kb in length. It is a globally recognized bacteria that have being used as models in several type of research that involve fermentation (Bertrani & Salvador, 1952). *E. coli* is easy to grow, able to live in room temperature and easy to prepare. The process of growing *E. coli* can be done in solid or liquid culture media that contain carbohydrates, vitamins, salts, proteins and nucleic acids (Bertrani & Salvador, 1952). According to Bertrani & Salvador (1952), *E. coli* can be directly grown using LB broth and LB agar.

According Alterthum & Ingram (1989), bacteria such as *E. coli* have the ability to metabolize all sugar constituents in lignocellulosic materials. Environment hardiness, broad substrate range and ability to grow well in mineral salts media are part of criteria for the selection *E. coli* as a platform organism for metabolic engineering (Zhou *et al.*, 2006). The modification of *E. coli*, by the insert of PET (production of ethanol) operon into the pyruvate formatelyase gene of *E. coli*, caused a change in gene of *E. coli* that disrupts to the terminal fumarate reductase gene of the succinate pathways, producing new strain (Walton, 2009). This stain was named *E. coli* K011 (Walton, 2009). The modified *E. coli* strain allow it to perform fermentation in a wide range pentose sugars such as xylose, as well as hexose sugars, to ethanol.

2.3 Bioethanol and Ethanol Productions

Biofuel is valuable resources that have being introduced in transportation sector. These liquid biofuels include ethanol, methanol, plant oils and methyl esters (Uriarte, 2010). The demand for biofuel is increasing lately as the number vehicle on the roads increases. Hence, the idea studying non-petroleum based fuels escalated as biofuel may provide a valuable route to resolving current issues on petroleum and keeping the price of energy at affordable level (Ogunniyi, 2005). Fossil fuel is obtain deep inside the earth, the formation is due to decomposition of plant and animal millions years ago. Biofuel are almost similar to fossil fuels, but it is only made up of decomposition of plant (Leen, 1996).

Bioethanol is a commonly known renewable fuel. It can be produced using fermentation processes that involve sugar and starch, that can be obtained from plant component such as decompose of grapes, corn, sugarcane, bananas and many more (Sticklen, 2008). According to Hygrell, (2006), combustion of bioethanol will produce clear and clean emissions. Engines running on bioethanol also has lower emission of solid particle, thus helping to reduce pollutant emission and limiting greenhouse gases (Hygrell, 2006).

2.4 Fed-batch Fermentation

Fed-batch fermentation is a process that has been commonly use for the production of bioethanol. There are many other types of processes used to carry out fermentation in several mode including batch fermentation, continuous fermentation, continuous fermentation with cell recycling, fed-batch, and repeated-batch culture (Yoshida *et al.*, 1973). Fed-batch process operate by feeding one or more nutrient to bioreactor during cultivation period, every 6, 12, 24, 36, 48, 60, 72 and 84 hours respectively, depending on experiment. The implementation feeding the nutrient, allowing the substrate been maintain and monitor in the fixed rate.

Fed-batch cultivation are also known as semi-batch culture. The advantages are basal medium support initial growth and production, fed medium prevent depletion of nutrients and ability to sustain the production phase (Fike, 2009). By using fed-batch process undesired effect in fermentation, such as substrate inhibition can be minimized. Besides that, the extension of process time and control of fermentation are also beneficial aspects of fed-batch operation (Kiran *et al.*, 2009).

2.5 High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography is a chromatography system that able to detect and analyze the presence of many biological compound such as cellulose, glucose, xylose and fermentation products such as ethanol, lactic acid and acetic acid in sample (Vincent *et al.,* 2011). HPLC also help assist in several other aspect such as absorption, partition, exchange of ion, exlusion and affinity chromatography which allow it to work faster and give better resolution result (Wilson & Walker, 2005). A microprocessor is a control the systems, that allow to run continuous chromatography separation without need to exchange the column.

The main concept of HPLC are based on chromatographic procedure, which is the mobile and stationary phase were use as separation method (Standardbase technique, n.d). In order to separate the sample mixture, HPLC will utilize the mobile phase and stationary phase form in liquid or solid (Standardbase technique, n.d). This component will be analyzed and dissolved in solvent flow through the chromatographic column under high pressure, hence result separated into various components (Standardbase technique, n.d). Based on previous analysis done by Lindsay *et al.* (1992), HPLC are reported to be relatively easier to perform compare to other chromatography procedures.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

The materials that were used in this study are:

1. Castor oils (Aldrich Chemical Co., Inc. India)
2. PBS buffer :
   i. Sodium Chloride (Merck, Germany)
   ii. Potassium Chloride (J.T. Baker, USA)
   iii. Sodium Hydrophosphate (Merck, Germany)
   iv. Potassium di-hydrogen phosphate (J. T. Baker , USA)
3. *Escherichia coli* K011
4. Distilled water
5. D-(+)-Glucose (Sigma, USA)
6. Luria broth (Sigma, USA)
7. Plate Count Agar (Merck, Germany)
8. Fermentation Broth : (Dowe and McMillan, 2008)
   i. 1X YP medium (liquid)
   ii. 1.5 g Yeast extract (CONDA, Spain)
   iii. 3.0 g Peptone (CONDA, Spain)
9. 1 M Citrate buffer
   i. 44g Citric Acid (Fisher, United Kingdom)
   ii. 86g Sodium citrate (QREC, Malaysia)

3.2 Methods

3.2.1 *Escherichia coli* K011 preparation

E. coli K011 culture preparation was done by growing overnight in 100 ml sterile LB broth placed in an incubator shaker at 37 °C with constant agitation at 120 rpm. Then, cell were harvested through centrifugation in 50 ml conical centrifuge tube (Vincent *et al.*, 2011).

3.2.2 Fermentation Broth

Fermentation broth was prepared in bottles with the mixing of all the reagent and medium such as YP solution with pre-determined glucose/xylose weight and *E. coli* K011 as fermentation agent in 500 ml bottle. The glucose/xylose concentration (w/v) were set at 5%, 10%, 20% and 30% respectively. One set of control without castor oil was prepared another set were added with 15 ml of castor oil. The fermentation broth was then incubated for four days. As for fed-batch procedure glucose/xylose were added at 0, 24 and 48 hours of fermentation process. Sampling period were set at 0, 6, 24, 48, 60, 72 and 84 hours respectively. The fed-batch fermentation preparation were set up by controlling agitation at 120 rpm and temperature of 37 °C respectively.

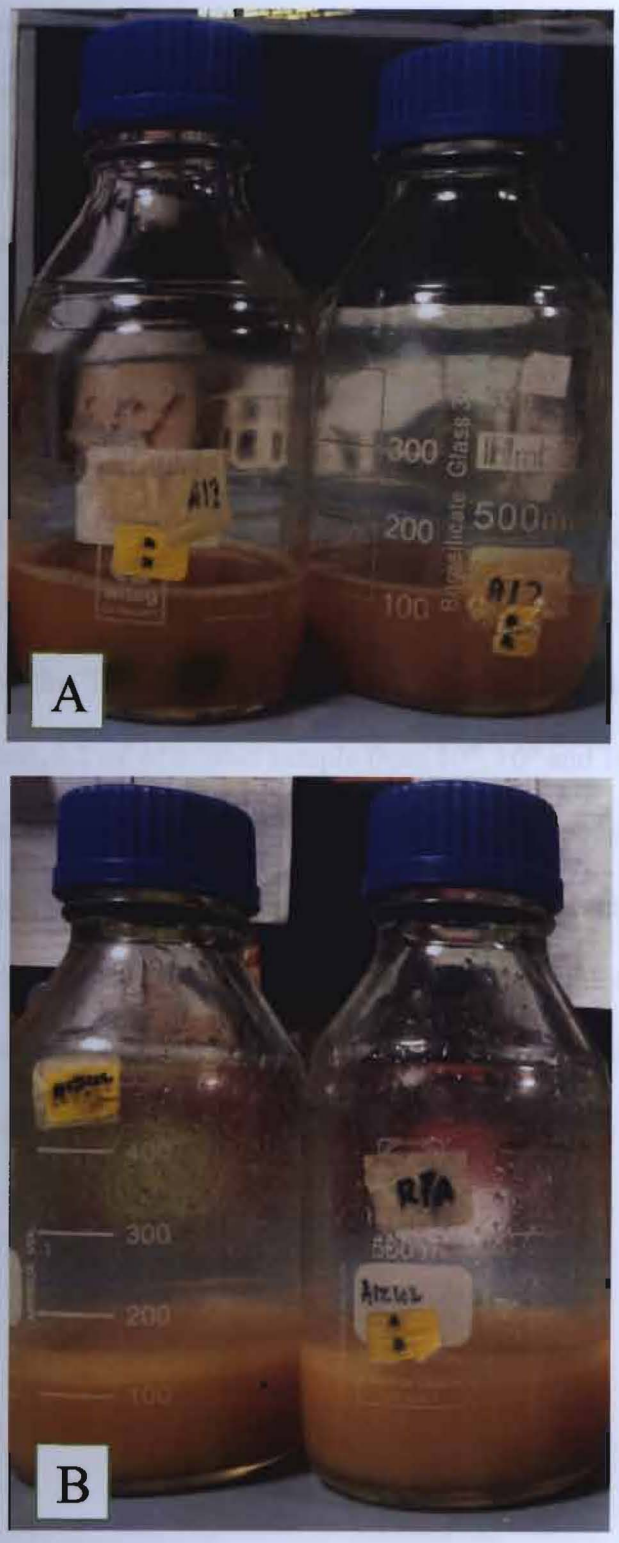

Figure 3: Fermentation broth A) control broth and B) with addition of castor oil in different glucose/xylose concentration.

3.2.3 Sample Processing

At 0, 6, 12, 24, 36, 48, 60, 72 and 84 hr of fermentation, 3 ml sample were obtained aseptically. One tube were used direct for plate count and others tubes were centrifuge at 14000 rpm for 3 minutes. Then it will be filtered using nylon syringe filter (0.45 μm) to obtain clear supernatant then kept in -20 °C before run HPLC analysis.

3.2.4 Viable cell count (CFU/ ml)

Viable cell method were done on plate count agar. Then 300 μl samples were obtain from the fermentation broth. Then 0.5 ml sample was transfer from first test tube to another test tube contain PBS buffer. The dilution factor will increases depend on observation after 24 hours (Figure 4). Next, 0.2 ml of diluted sample from 10^4, 10^5 and 10^6 test tube was pipetted out from the test tube and poured on to the surface of plate count agar prepared earlier. Sterile "hockey stick" were used to spread the diluted sample evenly on the surface of agar inside the plate. The agar was incubated and store in 37 °C for 24 hours. The number of colony forming units (CFU) for each plate was counted and number of bacteria in original suspension were calculated.